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Phosphatidylinositide Dependent Kinase Deficiency Increases Anxiety and Decreases GABA and Serotonin Abundance in the Amygdala

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Key Words
Anxiety • GABA • Serotonin • Amygdala • Behavior • PDK1 • PI3K • BDNF • Depression

Abstract
Pathological anxiety is paralleled by deficits in serotonergic and GABAergic neurotransmission in the amygdala. Conversely, anxiety disorders and depression may be reversed by brain-derived neurotrophic factor (BDNF). BDNF signaling involves Phosphatidylinositol 3-Kinase / 3-phosphoinositide-dependent protein kinase 1 (PI3K/PDK1). We thus hypothesized that impaired function of PDK1 might be associated with increased anxiety and concomitant neurotransmitter changes. Here we used the hypomorphic PDK1^{hm} mouse to investigate anxiety behavior in different settings: PDK1^{hm} mice differed from Wt littermates PDK1^{WT} in several behavioral measures related to anxiety and exploration, namely in the open field, dark-light box, O-maze and startle response. Further we analyzed the brain substrate underlying this phenotype and found significantly decreased GABA, taurine and serotonin concentrations in the amygdala and olfactory bulb of PDK1^{hm} mice, while BDNF and nerve growth factor (NGF) concentrations were not significantly different between PDK1^{hm} and PDK1^{WT} mice. These results suggest that impaired PI3K signaling in the PDK1^{hm} mouse reduces concentrations of GABA and serotonin in anxiety related brain regions and can serve as a molecular substrate for behavior indicative for anxious and depressive-like mood states.

Introduction

A large body of evidence from rodent studies indicates that the amygdala plays the central role in the acquisition and expression of conditioned fear [1] and recently it has been shown that fear learning in the amygdala involves brain-derived neurotrophic factor (BDNF) and Phosphatidylinositol 3-Kinase (PI3K) activation [2]. Studies in humans have largely confirmed hypotheses about fear conditioning from animal studies, where neuroimaging studies demonstrate that the amygdala is critical for emotional learning in fear conditioning paradigms [1, 3].

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The BDNF hypothesis of depression and anxiety postulates that a loss of BDNF is directly involved in the pathophysiology of depression and anxiety and its restoration may underlie the therapeutic efficacy of antidepressant treatments [4, 5]. BDNF affects neuronal growth, neurogenesis and memory in a PI3K dependent way [2, 6]. BDNF signaling involves phosphoinositide-dependent protein kinase 1 (PDK1) [7].

Accordingly, the PI3K signaling pathway has been shown to regulate hippocampal proliferation, differentiation and survival, to develop dendrite size and shape and to induce functional synapses in neuronal cells [8-10]. Both, pharmacological inhibition and genetic manipulation of PI3K-dependent signaling demonstrate that PI3K signals via PDK1 [11]. PDK1 is a direct downstream effector of PI3K [11] and plays a central role in activating other downstream processes, including cell proliferation, survival and differentiation [12].

On the neurotransmitter level, especially serotonin (5-HT) and GABA have been shown to powerfully regulate the neuroanatomical circuit that mediates fear in anxiety disorders [13].

To explore the impact of PDK1 signaling on transmitter concentrations in amygdala and on behavior, gene targeted mice were analyzed in the present study. As the PDK1 knockout mouse is not viable [14], mice with suppressed PDK1 activity to some 10-25 % ($PDK1^{hm}$) [14] were compared to their age and sex matched wild type littermates ($PDK1^{WT}$).

Materials and Methods

Animals

The generation and basic properties of PDK1 hypomorphic mice have been described earlier [14, 15]. Genotyping was made by PCR on tail DNA using PDK1 and neo-R-specific primers as previously described [14]. Mice had free access to standard mouse diet (Altromin, Langen, Germany) and tap water. All animal experiments were conducted according to the guidelines of the American Physiological Society and the German law for the welfare of animals and were approved by the local authorities. For the present study 12 PDK1 hypomorphic mice ($PDK1^{hm}$) and their wild type littermates (n=16; $PDK1^{WT}$) were used.

Housing and handling

One week before the experimental period, animals were transferred to the Institute of Anatomy in Zuerich where they were single housed in standard type II mouse cages and maintained at a 12:12 h inverted cycle with lights on between 8 p.m. and 8 a.m. Standard mouse chow, water and nesting material were available ad libitum. Behavioral testing occurred between 8 a.m. and 8 p.m. Only one type of experiment was done on the same day and the home cage rack was brought to the test room at least 30 min before each experiment and dry surfaces of apparatus were thoroughly cleaned with 70 % ethanol before releasing the animal. Experiments extended over a total of 6 weeks, average age was 5.6 months at the beginning and 7.5 months at the end of the testing period.

Behavioural studies

For data acquisition, animals were video tracked at 4.2 Hz and 576x768 pixel spatial resolution using a Noldus EthoVision 3.0 system (Noldus InformationTechnology, Wageningen, The Netherlands, www.noldus.com). Raw data were transferred to the public domain software Wintrack 2.4 [16] (www.dpwolfer.ch/wintrack) for further analysis.

Tests were done in the following order: open-field, LD-box, O-maze, emergence test, object exploration, and acoustic startle response profile. Experiments were performed with diffuse indirect room light produced by 440W bulbs, adjusted to yield approximately 12 lux in the center of the experimental arena. The only exception was the LD-box test where full room light was switched on to obtain approximately 500 lux in the lit chamber. The experiments have been performed as described previously in detail [17].

For open-field the round open-field arena had a diameter of 150 cm, a white plastic floor, and 35 cm high sidewalls made of white polypropylene. Each subject was released near the wall and observed for 10 min. The same procedure was repeated the following day, resulting in a total observation time of 20 min [18].

For the light-dark box a 20x30 cm transparent Perspex chamber (20 cm high) under direct room light was connected by a 7.5x7.5 cm aperture to a 20x15x20 cm polyvinyl-chloride dark box. Each subject was released in the middle of the lit compartment and observed for 5 min [19].

For O-maze a 5.5 cm wide annular runway was constructed using grey plastic. It had an outer diameter of 46 cm and was placed inside the above open-field arena 40 cm above the floor [17, 20]. The two opposing 90° closed sectors were protected by 16 cm high inner and outer walls of grey polyvinyl-chloride, while the remaining two open sectors had no walls. Animals were released in one of the closed sectors and observed for 10 min.

For the emergence test the procedure was modified after Dulawa et al. [21]. Frames of non-reflective aluminium (37 cm high) were used to partition the above open-field into four square 50x50 cm arenas, allowing for concurrent observation of 4 animals. Each arena had a 12x8x4 cm plastic home box with an aperture of 8x4 cm, positioned in a corner at 5 cm from the nearest walls, with the aperture facing away from the wall. 24 h prior to testing, a thoroughly cleaned home box was placed in the home cage of each test subject. The next day, test subjects and home boxes were introduced into the arenas and observed for 30 min.

For the object exploration test, the procedure was modified after Dulawa et al. [21]. Arenas were the same as for the emergence test, but without the home box. The novel object was a 12x4 cm semi-transparent 50 ml Falcon tube positioned

vertically in the center of the arena and fixed with adhesive tape. Each subject was observed for 30 min in the empty, cleaned arena. Then, the novel object was introduced and observation continued for another 30 min.

For acoustic startle response, testing was conducted using a Hamilton-Kinder SM100 startle monitor system (www.hamiltonkinder.com, Poway, CA). The rectangular animal restrainer (3.8x8.8 cm) was made of clear Perspex and had an adjustable ceiling which was set to prevent the animal from rearing. The restrainer rested on a sensing plate which carried a piezoelectric accelerometer at its bottom. The unit was mounted on a heavy metallic base plate by four mounting pins and enclosed in a sound-attenuated ventilated cabinet (internal dimensions 29x29x18 cm). The loudspeaker was located 22 cm above the animal and produced white noise pulses. A microcomputer interface controlled the loudspeaker and performed an A/D conversion of the signals from the accelerometer. Signal calibration was done using a Newton impulse calibrator. Sound levels were verified using a digital sound level meter (Radio Shack). The background noise level inside the closed cabinet was maintained at 70 dB. Subjects were placed in the thoroughly cleaned and dried restrainer and left undisturbed for 5 min before the session began. In total, 66 trials were presented. Nine different sound levels (dB) were used: 64, 68, 72, 76, 80, 90, 100, 110, and 120 [22]. Each stimulus was 40 ms and presented 6 times in pseudorandom order such that each sound level occurred once within a block of 9 trials. The series began and ended with 6 presentations each of the 120 dB stimulus. The average intertrial interval was 15 s (ranging from 10 to 20 s). The startle response was recorded for 250 ms (measuring the response every 1-ms) starting with the onset of the startle stimulus and the maximum amplitude was used as the dependent variable.

Dissection of the brains and homogenization procedure

Mice were sacrificed 7 days after the last behavioral experiment. After decapitation, the brains were rapidly removed, immediately frozen on dry ice and stored at -80 °C until use. For dissection the frozen brains were placed on a cold plate providing a temperature between -10 and -12 °C. At this temperature the brain tissue was in a semi-frozen state with well-preserved anatomical structure. The amygdala/piriform cortex was cut out using the rhinal fissure as landmark. The olfactory bulb was dissected along the basis of the frontal cortex. Each tissue sample was homogenized by ultrasonication in 40-80 volumes of distilled water. For the determination of NGF and BDNF protein 150 µl of the homogenate were added to an equal volume of buffer containing 0.1 M Tris-HCl, pH 7.0, 0.4 M NaCl, 0.1% NaN_3, and a variety of protease inhibitors ("complete"-Protease Inhibitor Cocktail Tablets, Roche Diagnostics GmbH, Penzberg, Germany). For the analysis of monoamines and metabolites 150 µl of the homogenate were added to an equal volume of 0.2 M perchloric acid and centrifuged at 25,000 *g* for 20 min at 4 °C. 5-HT, 5- hydroxyindoleacetic acid (5-HIAA), and the catecholamines noradrenaline (NA) and dopamine (DA) were measured in the supernatant [23]. For amino acid analysis 20 µl of the homogenate were added to 80 µl methanol and centrifuged at 25,000*g* for 20 min at 4 °C. The supernatant was diluted 1:30 with distilled water.

Determination of NGF and BDNF protein

Endogenous NGF levels in the thawed homogenates were determined by a fluorometric two-site enzyme immunoassay (ELISA) which has been described in detail elsewhere [23, 24]. The mean recovery of mouse NGF (125 pg/ml) added to the homogenate ranged from 60 % to 90 %. NGF content was expressed as equivalents of mouse 2.5S NGF. The detection limit of the assay was 0.25 pg/ml.

Endogenous levels of BDNF were measured in the thawed homogenates using commercial ELISA kits in principle according to the manufacturer's instructions (Promega, Inc.) but adapted to the fluorometric technique also used for NGF determination [25, 26]. BDNF content was expressed as equivalents of recombinant human BDNF. The detection limit of the assay was 1 pg/ml. Each sample (namely each tissue dissected and homogenized as described above) was consecutively processed for quantification of each neurotrophin, i.e., NGF and BDNF [25, 26]. Determinations of recovery, and specific and unspecific neurotrophin binding (the latter against mouse IgG_1 obtained from MOPC 21) involved quadruplicate fluorescence determinations for each tissue sample. The neurotrophin levels were expressed as pg/mg tissue (wet weight). In order to minimize the influence of unavoidable variances between experiments, neurotrophin levels from animals were normalized as a percentage of wildtypes serving as control, always being included in the same experiment [23].

Determination of monoamines and metabolites

5-HT and 5-HIAA were analyzed as described previously using high-performance liquid chromatography (HPLC) with electrochemical detection [27]. NA and DA were measured by HPLC with electrochemical detection after extraction to alumina, according to Felice et al. [28] with minor modifications [29].

Determination of amino acids

Glutamate, γ-amino butyric acid (GABA), and taurine were determined using methods described previously [30]. In brief, amino acids were precolumn derivatized with *o*-phthalaldehyde-2-mercaptoethanol using a refrigerated autoinjector and then separated on a HPLC column (ProntoSil C18 ace-EPS) at a flow rate of 0.6 mL/min and a column temperature of 40 °C. The mobile phase was acetonitrile 50 mmol/mL sodium acetate solution pH = 5.7 in a linear gradient from 5 % to 21 % acetonitrile. Derivatized amino acids were detected by their fluorescence at 450 nm and excitation at 330 nm.

Statistics

Data were analyzed using a two-way factorial ANOVA design with genotype as between subject factor. Where appropriate, the model was complemented by within subject factors to explore the dependence of genotype effects on place, time, and stimulus intensity. One-sample t-tests were used to compare group means with hypothesized values.

Table 1. Brain and body weight. (16 Wt, 12 KO, cohort 1 4.9-11.8 months), factorial ANOVA. Type I error p-values are shown for all effects if <0.1, followed by estimated effect sizes shown as partial omega squared, the proportion of variance accounted for by genotype, if only this factor were in the design (range 0 to 1.0). Up and down arrows indicate direction of mean differences if type I error p<0.25.

brain weight	genotype		
average body weight (g)	F(1.24)=39.9	↓P<0.0001	0.58
fresh brain weight (g)	F(1.24)=476.4	↓P<0.0001	0.94
brain / body weight (%)	F(1.24)=0.1	ns	

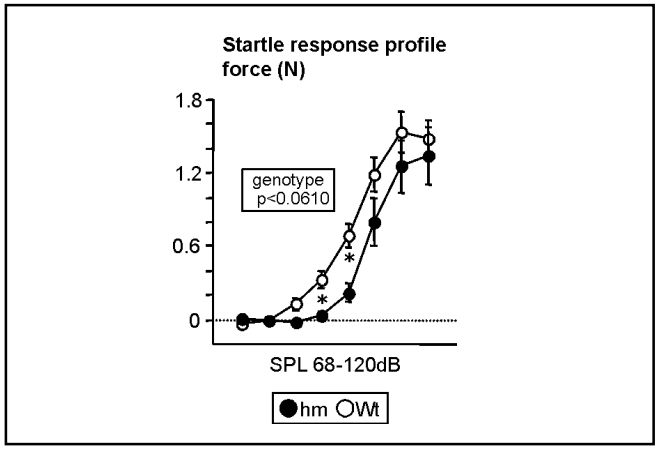

Fig. 1. Acoustic startle response profile (16 Wt, 12 PDK1^{hm}, 4.1 - 10.9 months). Startle response amplitude to stimuli of 68, 72, 76, 80, 90, 100, 110, 120 dB. The response profile of PDK1^{hm} tended to be shifted to the right, indicating a possible threshold increase (genotype F(1.24) = 3.9 p<0.0610, stimulus F(7.168) = 54.0 p<0.0001, genotype x stimulus F(7.168)=1.2 ns; * significant genotype effect at individual SPL after correction for multiple comparisons according to false discovery rate control procedure of Benjamini and Hochberg.

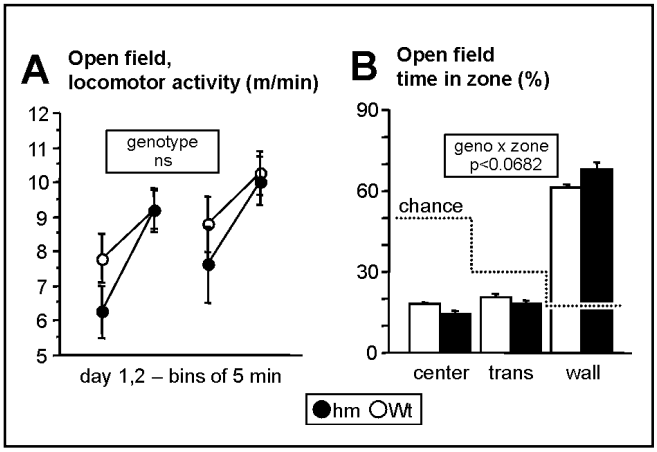

Fig. 2. Open field (16 Wt, 12 PDK1^{hm}, 3.7 - 10.6 months). A. Distance traveled during the first and second 5 minutes during the first and second day. Even though the plot suggests that PDK1^{hm} were less active than Wt during the first 5 min on each day, the time course of activity is statistically indistinguishable between groups (genotype F(1.24) = 0.8 ns, time F(3.72) = 14.9 p<0.0001, genotype x time F(3.72) = 1.3 ns). B. Time spent in the center, transition and wall zone. Both groups avoided the center and transition zones in favor of the wall zone. PDK1^{hm} tended to prefer the wall zone slightly more than controls. (zone F(2.48) = 308.7 p<0.0001, genotype x zone F(2.48) = 2.8 p<0.0682).

Where possible, ANOVA main effects were verified using nonparametric tests which produced similar results. Statview version 5.0 (SAS Institute, Cary, North Carolina, www.statview.com) was used for all statistical calculations. The software Sigma Stat was used for the statistical analysis of data concerning neurotrophin and metabolite concentrations. For statistical analysis of the biochemical data the Student's t-test was applied.

Results

Body and brain weight

PDK1^{hm} mice appeared healthy, but they were considerably smaller than their wild type littermates PDK1^{WT} (16 PDK1^{WT}, 12 PDK1^{hm}, 4.1-10.9 months). Body weight of PDK1^{hm} mice was significantly lower in PDK1^{hm} than in PDK1^{WT} mice (p<0.0001, Table 1), which was the case also for fresh brain weight (p<0.0001,

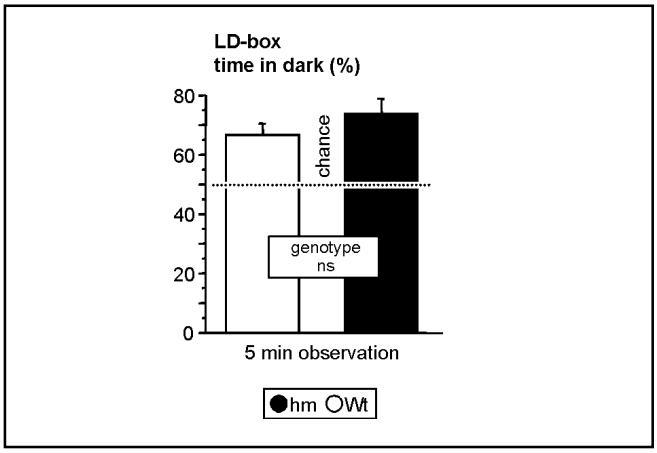

Fig. 3. Light/dark box (16 Wt, 12 PDK1^{hm}, 3.8 - 10.7 months). Time spent inside dark compartment. Mice showed a clear preference for the dark compartment (genotype F(1.24) = 2.1 ns, one-sample t-test against chance value of 50 % t(27) = 5.9 p<0.0001).

Fig. 4. O-maze (16 Wt, 12 KO, cohort 1 3.8 - 10.7 months). A. Time spent on open / closed sectors and in the transition zone. Both groups strongly avoided the open sectors. KO spent more time on the closed sector than Wt who preferred to stay in the transition zone. (zone F(2,48) = 0.4 p<0.0001, genotype x zone F(2,48) = 5.7 p<0.0062; genotype by zone: open F(1,24) = 0.5 ns, transition F(1,24)=6.9 P<0.0148, closed F(1,24)=6.0 p<0.0224). B. Number of head dips. KO mice performed less protected head dips. The number of unprotected head dips did not differ from Wt (genotype F(1,24) = 2.1 ns, zone F(1,24) = 81.3 p<0.0001, genotype x zone F(1,24) = 9.0 p<0.0061; genotype by zone: unprotected F(1,24) = 0.1 ns, protected F(1,24) = 5.1 p<0.0337).

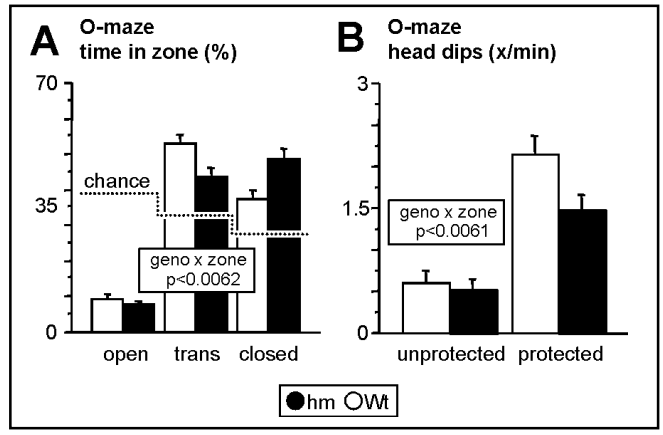

Table 1). The weight of PDK1^{hm} mice amygdala was significantly less in PDK1^{hm} than in PDK1^{WT} mice (17.73 ± 0.58 mg/1000µl; 23.63 ± 0.51 mg/1000µl; p<0.001), which was the case also for the olfactory bulb (17.68 ± 0.39 mg/1000µl; 24.18 ± 0.55 mg/1000µl; p<0.001). The relation between reduction of brain and body weight was not significantly different between PDK1^{hm} and PDK1^{WT} mice (Table 1).

Behavioral studies

In the acoustic startle response profile, the response profile of PDK1^{hm} tended to be shifted to the right, indicating a threshold increase (genotype F(1.24) = 3.9 p<0.0610, stimulus F(7.168) = 54.0 p<0.0001, genotype x stimulus F(7.168) = 1.2 ns) (Figure 1).

In the open field, no overt differences of spontaneous behavior have been detected between genotypes, but there was a trend for PDK1^{hm} mice to spend less time in the center field (Figure 2).

In the LD-box, light-dark transitions were significantly less frequent in PDK1^{hm} than in PDK1^{WT} mice, without significant differences between genotypes in the total amount of time spent in the dark (Figure 3).

On the O-maze, PDK1^{hm} spent more time than PDK1^{WT} mice inside the protected sectors and displayed less risk assessment at the transition between closed and open sectors (Figure 4).

In the emergence test and object exploration (open field with home box and additionally a novel object) PDK1^{hm} mice showed, as compared to PDK1^{WT} mice, increased activity towards the object and spent more time inside the home box, but showed normal exploratory activity while outside the box (Figure 5, 6).

Neurotransmitter concentrations

In the amygdala, PDK1^{hm} mice had, as compared to PDK1^{WT} mice, lower 5-HT concentrations (Table 2,

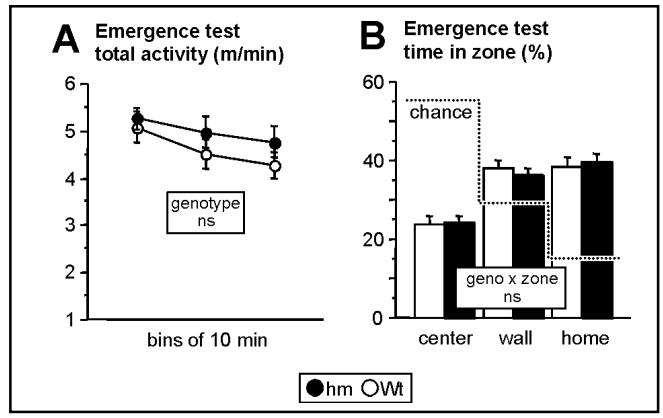

Fig. 5. Emergence test (16 Wt, 12 PDK1^{hm}, 3.9 - 10.8 months). A. Distance moved in 10 min. PDK1^{hm} showed the same initial activity as Wt but female PDK1^{hm} habituated less than Wt (genotype F(1.24) = 0.5 ns, time F(2.48) = 14.5 p<0.0001, genotype x time F(2.48) = 2.5 p<0.0895). B. Time spent in the center, wall and home zone. Both groups avoided the center zone and preferred to stay in the zone surrounding the home box (zone F(2.48) = 18.8 p<0.0001 genotype x zone F(2.48) = 0.8 ns).

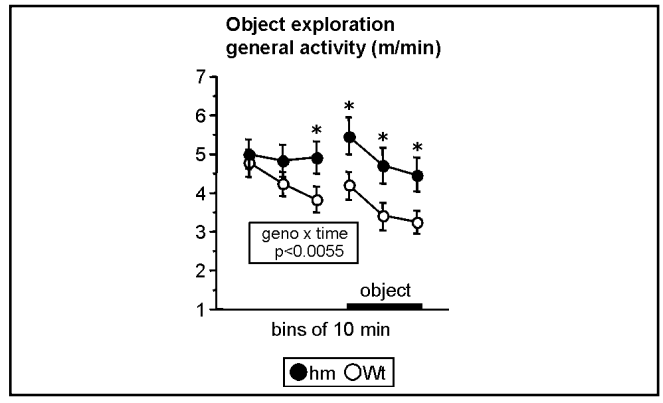

Fig. 6. Object exploration (16 Wt, 12 PDK1^{hm}, 3.9 - 10.8 months). Distance moved in 10 min. PDK1^{hm} did not habituate during period before insertion of the object and were more active than Wt while the object was present (genotype F(1.24) = 3.6 p<0.0690, time F(5.120) = 10.9 p<0.0001, genotype x time F(5.115) = 3.5 p<0.0055, * genotype p<0.05 for split ANOVA at single time points).

	WT (n=16)	PDK1 (n=12)	Significance
5-HT	1122.3 ± 36.6	1019.4 ± 20.7	p = 0.034
5-HIAA	805.6 ± 38.0	885.7 ± 62.9	n.s.
5-HIAA/5-HT	0.668 ± 0.032	0.805 ± 0.063	p = 0.046
NA	383.5 ± 19.5	461.5 ± 23.8	p = 0.017
DA	285.2 ± 29.8	258.9 ± 39.5	n.s.
GABA	3.1 ± 0.1	2.85 ± 0.05	p = 0.015
Glutamate	11.94 ± 0.26	12.1 ± 0.5	n.s.
Taurine	12.8 ± 0.2	11.87 ± 0.35	p = 0.023
NGF	5.51 ± 0.52	5.6 ± 0.4	n.s.
BDNF	20.2 ± 2.4	24.4 ± 3.3	n.s.

Table 2. Neurotransmitter and neurotrophin concentrations in the amygdala. Data are presented as means ± SEM. The levels of 5-HT, 5-HIAA, NA, DA, NGF and BDNF are expressed as pg/mg tissue, the levels of GABA, glutamate and taurine as pmol/mg tissue.

	WT (n=16)	PDK1 (n=12)	Significance
5-HT	561.6 ± 16.9	614.0 ± 31.1	n.s.
5-HIAA	796.7 ± 25.5	945.9 ± 75.6	P = 0.047
5-HIAA/5-HT	1.314 ± 0.039	1.418 ± 0.078	n.s.
NA	195.3 ± 12.0	243.1 ± 12.0	P = 0.010
DA	248.5 ± 12.1	263.4 ± 17.6	n.s.
GABA	9.54 ± 0.19	8.87 ± 0.18	P = 0.019
Glutamate	8.0 ± 0.2	7.9 ± 0.3	n.s.
Taurine	18.66 ± 0.37	17.84 ± 0.94	P = 0.021
NGF	10.54 ± 1.19	11.75 ± 1.94	n.s.
BDNF	8.71 ± 1.24	8.87 ± 1.59	n.s.

Table 3. Neurotransmitter and neurotrophin concentrations in the olfactory bulb. Data are presented as means ± SEM. The levels of 5-HT, 5-HIAA, NA, DA, NGF and BDNF are expressed as pg/mg tissue, the levels of GABA, glutamate and taurine as pmol/mg tissue.

p = 0.034) and an increased 5-HT turnover as indicated by the molar ratio of 5-HIAA to 5-HT (Table 2, p = 0.046). The NA content was increased in PDK1^{hm} mice (Table 2, p=0.017). GABA and taurine concentrations in the amygdala were lower in PDK1^{hm} than in PDK1^{WT} mice (Table 2, p = 0.015, p = 0.023), whereas glutamate and DA were not significantly different between genotypes. BDNF and NGF concentrations in the amygdala were not significantly different between PDK1^{hm} and PDK1^{WT} (Table 2).

In the olfactory bulb, 5-hydroxyindoleacetic acid concentrations were higher in PDK1^{hm} mice than in PDK1^{WT} mice (Table 3, p = 0.047) but 5-HT concentrations were not significantly different between PDK1^{hm} and PDK1^{WT}. NA concentration was significantly larger in PDK1^{hm} than in PDK1^{WT} mice (Table 3, p = 0.010). In the olfactory bulb GABA and taurine concentrations were lower in PDK1^{hm} than in PDK1^{WT} mice (Table 3, p = 0.019, p = 0.021). Glutamate and DA concentrations in the olfactory bulb were not significantly different between PDK1^{hm} and PDK1^{WT} mice. Also BDNF and NGF concentrations in the olfactory bulb were not significantly different between PDK1^{hm} and PDK1^{WT}.

Discussion

This first behavioral examination of the PDK1^{hm} phenotype revealed increased anxiety behavior in several different tests, i.e. open field, light dark box and zero maze test, which was accompanied by changes of GABA, NA and 5-HT concentrations in the amygdala and olfactory bulb.

As reported earlier [14], body weight in PDK1^{hm} was significantly reduced, which was proportional to decreases in brain weight. This decrease appears to be primarily due to decreased cell volume rather than reduction of cell number [14]. Amongst other factors cell volume and brain metabolism are determined by cellular uptake of amino acids [31]. The influence of PDK1 deficiency on brain neurotransmitter abundance and anxiety behavior could thus be related to altered amino acid transport. PDK1 activates the serum and glucocorticoid inducible kinase and protein kinase B PKB/Akt isoforms [32], which in turn have been shown to regulate a wide variety of transporters [33]. Previous experiments in PDK1^{hm} mice indeed revealed a decreased activity of glucose transporters [34] and amino acid transporters [35]. Moreover, an increased fluid and food intake has been reported in PDK1^{hm} mice, while urinary excretion of glutamate and tryptophan, but not 5-hydroxytryptophan is increased in PDK1^{hm} mice [35]. However, plasma concentrations of these cerebrally relevant amino acids (glutamate, tryptophan, glycine) in PDK1^{hm} mice did not differ significantly from those of PDK1^{WT} mice [35]. Similarly, DA and glutamate concentrations in amygdala and olfactory bulb were not significantly different between PDK1^{hm} and PDK1^{WT} mice (Table 2, 3).

In our study, PDK1^{hm} mice differed from Wt littermates in several behavioral measures related to anxiety and exploration (Figure 2-4). In the emergence test (open field with home box), PDK1^{hm} spent more time inside the home box, but showed normal exploratory activity while outside the box (Figure 5, 6). This observed normal exploratory activity in the emergence test does not fit to increased anxiety behavior but might be explained by agitation [36-38].

Increased anxiety behavior in PDK1^{hm} mice can be linked to decreased activation of the PI3K/Akt pathway, which has been shown to contribute to mechanisms of synaptic plasticity and memory consolidation by promoting

cell survival and synapse number [9, 39]. After activation of PI3K, PDK1 is activated leading to phosphorylation of PKB/Akt. Activated PKB/Akt phosphorylates intracellular substrates, promoting cell survival by inhibiting apoptosis through several targets, including Forkhead transcription factors and mammalian target of rapamycin [9]. One of the essential functions of PKB/Akt is the phosphorylation of glycogen synthase kinase 3 GSK-3 causing its inactivation and activation of cAMP response element binding protein (CREB) [10]. Decreased CREB function in the central amygdala has been involved in anxiety behavior [40, 41] and a decreased CREB phosphorylation after BDNF antisense infusion has been found [42], which might be attributable to decreased PI3K activity. Recently, it has been shown that fear learning in the amygdala involves BDNF and PI3K activation [2].

A role for the PI3K signaling cascade in anxiety behavior is plausible as several factors influencing anxiety behavior act via PI3K. Recently, it was demonstrated *in vivo* that anxiolytic cannabinoids activate the PI3K/Akt pathway and negatively regulate GSK-3β activity in the mouse brain [43]. Also insulin-like growth factor-1 (IGF-I) and estrogen have been shown to disrupt anxiety behavior and to activate PI3K [44-46]. Moreover exercise, with its well known anxiolytic properties, activates PI3K pathway [47, 48]. BDNF has also been related to anxiety behavior [49, 50] and fear conditioning especially in the amygdala [2, 51, 52] and BDNF acts via PI3K [53, 54]. In this context, we expected a compensatory upregulation of BDNF and NGF in PDK1^{hm} which was, however, not observed (Table 2, 3). It should be kept in mind that PI3K signaling in PDK1^{hm} mice is not completely abrogated but PDK1 activity is suppressed by up to 80 % [14]. The residual PDK1 activity may be sufficient to prevent upregulation of BDNF. In any case, our findings of increased anxiety connected with deficient PI3K signaling, suggest that BDNF could influence anxiety at least in part via a PI3K dependent mechanism.

Moreover, several factors regulating PI3K activity might converge and exert additive effects, or, if absent, increase vulnerability. In line with this hypothesis heterozygous neuregulin 1 mice are more sensitive to behavioral effects of cannabinoids [55]. Additive genetic effects of BDNF and 5-HT transporters are dependent on estrogen modulation, thereby estrogen seems to compensate BDNF deficiency regarding behavior and brain monoamine levels [56]. Moreover, running exercise- and antidepressant-induced increases in growth and survival-associated signaling molecules are IGF-dependent [57].

Interestingly, the increased anxiety behavior in PDK1^{hm} mice was accompanied by a significant decrease of GABA, taurine and 5-HT in the amygdala, suggesting an involvement of these neurotransmitters in the determination of behavioral disturbances (Table 2). Moreover, 5-HT turnover was increased in the amygdala and seemed to be also increased in the olfactory bulb, suggested by increased concentration of 5-HIAA (Table 2, 3). Also in the olfactory bulb a decrease of GABA and taurine has been observed (Table 3). In both examined brain regions NA concentrations were increased (Table 2, 3).

A large body of evidence from rodent and human neuroimaging studies indicates that the amygdala plays a critical role in the acquisition and expression of fear behaviour [1, 3], which may explain that neurotransmitter changes in anxious PDK1^{hm} occur in this brain region. Also the olfactory bulb has been implicated in anxiety behavior [58]. Olfactory bulbectomy has been suggested as an animal model of comorbid anxiety and leads to hyperexcitability of amygdala neurons [58, 59]. Indeed, olfactory information converges in the amygdala, where the involvement of PI3K signaling has recently been observed in fear learning [2].

A decreased GABA concentration in amygdala and olfactory bulb fits well to the concept that GABA is the principal inhibitory neurotransmitter in the mammalian brain and its receptors are key control elements of anxiety states. The concept is based on the anxiolytic properties of benzodiazepines, which act as allosteric GABA receptor agonists [60]. Recently, first evidence for a control of GABAergic function through PI3K-mediated pathways has been reported [61]. Moreover, the additional observation of decreased taurine in both brain areas as observed in PDK1^{hm} mice is in line with the role of taurine as an anxiolytic-like amino acid after single and repeated administration and its modulation of amygdala associated anxiety [62, 63].

5-HT is believed to be the most relevant neurotransmitter in mediating anxiety [64]. Therefore, decreased 5-HT concentrations in the amygdala as found in our PDK1^{hm} mice might well explain increased anxiety behavior. The amygdala is overactive in patients with anxiety disorders and chronic administration of 5-HT reuptake inhibitors normalize amygdalar activity and are the treatment of choice in anxiety disorders [65-67].

The observed decreases of taurine concentrations in amygdala and olfactory bulb of PDK1^{hm} mice are similarly in line with increased anxiety behavior in PDK1^{hm} mice. In rats taurine microinjections in the

amygdala have been shown to reduce anxiety behavior [63] and an anxiolytic effect of taurine (i.p.) was also observed in mice [62].

The present study does not address the mechanisms underlying the influence of PDK1 on neurotransmitter levels. It is noteworthy, though, that PI3K signaling has been shown to participate in the regulation of GABA transport [68, 69], and the taurine transporter [68, 70-72]. Kinases activated by PDK1 include the serum and glucocorticoid inducible kinase (SGK) and protein kinase B PKB/Akt isoforms, which have been shown to regulate a wide variety of channels and transporters including glutamate transporters and glutamate receptors [33, 73-82]. Moreover, SGK and PKB phoshorylate and thus inhibit glycogen synthase kinase GSK3β [83], which plays a pivotal role in the regulation of behaviour [84-86]. Reduced locomotion reminiscent of the PDK1 hypomorphic mouse has been observed in SGK3 deficient mice [17].

In conclusion, we postulate that decreased PI3K signaling in the PDK1^{hm} mouse may provoke anxiety behavior with alterations of neurotransmitter concentrations in the amygdala and olfactory bulb, implicating a role of PI3K pathway in anxiety behavior. Our present study indicates that decreased PI3K signaling in PDK1^{hm} mice might be an animal model of increased anxiety, characterized by decreased GABA and 5-HT concentrations in the amygdala.

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